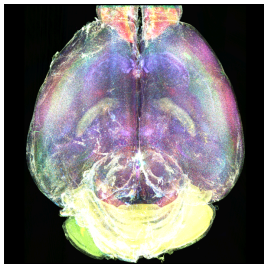
Feb 18, 2026 Version 2

# mFISH3D-2.0 V.2



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**Protocol status:** Working
**We use this protocol and it's working**

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## Abstract

The protocol is for the multiplexed in situ hybridization in 3D (mFISH3D-2.0) in an adult mouse brain / a block of a human brain. A comprehensive upgrade of old mFISH3D (https://www.protocols.io/view/mfish3d-kqdg3pjxql25/v1), representing a new generation rather than a simple extension. See our manuscript "Artificial intelligence-driven whole-brain cell mapping with highly multiplexed *in situ* hybridization" (peer-reviewed: https://doi.org/10.1016/j.neuron.2025.12.027, BioRxiv: https://doi.org/10.1101/2025.08.18.670857)

## Guidelines

See safety data sheets for proper chemical handling, precautionary measures, and waste disposal.
Obey all local regulations/guidelines for handling and disposal of used reagents and solutions containing reagents mixed in.

## Materials

To prepare a lithium maleate buffer, add lithium hydroxide to a maleic acid solution until the pH reaches 7.0. It is recommended to prepare a 1 M lithium maleate buffer stock solution.

**Material table**

| | A | B | C |
|---|---|---|---|
| | 16% PFA | Electron Microscopy Sciences | Cat. # 15710 |
| | 10X PBS | Invitrogen | Cat. # AM9625 |
| | Proteinase K | Ambion | Cat. # AM2544 |
| | Methanol | Fisher Scientific | Cat. # A412SK |
| | Formamide | Ambion | Cat. # AM9342 |
| | Poly(ethylene Glycol) 20,000 | Sigma | Cat. # 95172 |
| | Maleic acid | Sigma | Cat. # M0375 |
| | Lithium hydroxide | Sigma | Cat. # 442410 |
| | Sodium chloride | Millipore | Cat. # 567440 |
| | Lithium chloride | Sigma | Cat. # 213233 |
| | Hydrogen peroxide solution | Sigma | Cat. # 516813 |

| | A | B | C |
|---|---|---|---|
| | Benzyl alcohol | Sigma | Cat. # 24122-1L |
| | Benzyl benzoate | Sigma | Cat. # W213802-5KG-K |
| | Benzyl acetate | Sigma | Cat. # B15805 |

## Safety warnings

Formamide:
Handle with proper attire including gloves and eye protection. Work under fume hood when handling solution and dispose of waste appropriately.
Suspected of causing cancer.
May damage fertility or the unborn child.
May cause damage to organs (Blood) through prolonged or repeated exposure if swallowed.

Formaldehyde/paraformaldehyde/formalin solution (PFA):
Handle with proper attire including gloves and eye protection. Work under fume hood when handling solution and dispose of waste appropriately.
May cause cancer.
Toxic if swallowed, in contact with skin or if inhaled.
Causes severe skin burns and eye damage.
May cause an allergic skin reaction.
May cause respiratory irritation.
Suspected of causing genetic defects.
Causes damage to organs (Eyes).

Methanol:
Handle with proper attire including gloves and eye protection. Work under fume hood when handling solution and dispose of waste appropriately.
May be fatal or cause blindness if swallowed. Vapor harmful. Flammable liquid and vapor. Harmful if swallowed, inhaled, or absorbed through the skin. Causes eye, skin, and respiratory tract irritation. May cause central nervous system depression. Cannot be made non-poisonous.

## Before start

Before starting the staining, carefully design the combination of genes and the choice of dyes. We do not recommend using Alexa 488, as it cannot produce a good signal-to-noise ratio due to spectrum shifts in BABB. Instead, we suggest using Alexa 546, 647, and 750.
Alexa 750 is very useful if your microscope is equipped with an appropriate excitation laser (e.g., 785 nm), but the gene expression must be strong because of the limited sensitivity of the CMOS sensor at this wavelength range.
Alexa 647 is the most versatile dye and should always be your first choice. Alexa 546 also works well, though not as effectively as Alexa 647 due to higher autofluorescence. Based on our experience, it is not necessary to validate your probes on 2D sections, as probes that fail in 3D typically also fail in 2D, and vice versa.
If you need to perform triple staining, we recommend the following setup:

- Alexa 750: gene with the highest expression
- Alexa 647: gene with the lowest expression
- Alexa 546: gene with intermediate expression

Triple staining is commonly performed, but you should expect some trial and error (whether in 2D or 3D!). Before attempting triple staining, it is best practice to stain each gene individually to carefully assess potential crosstalk between channels and the impact of autofluorescence. It is always a good idea to search online to see whether your gene of interest has been examined by ISH in other studies using the same target tissue. For mouse brain samples, the Allen Brain Institute (**https://mouse.brain-map.org/**) provides an extensive resource. You may also consult public single-cell RNA sequencing datasets to determine whether your gene of interest is expressed in your tissue of choice. If no prior information is available, stick with single-color imaging using Alexa 647 at first. Otherwise, you will be in trouble determining whether staining failure results from methodological issues or simply because you are imaging a difficult target, such as a low-expression gene. If you are imaging tissue with strong autofluorescence (such as human tissue), we highly recommend imaging autofluorescence at the available channel. This will help you determine whether your ISH “signal” is a true signal or due to autofluorescence.

We use HCR v3, not GOLD, in the published study. You can purchase HCR v3 using the following link (https://store.molecularinstruments.com/new-kit/v3/rnafish)

Lithium maleate buffer is not commercially available. To prepare a lithium maleate buffer, add lithium hydroxide to a maleic acid solution until the pH reaches 7.0. It is recommended to prepare a 1 M lithium maleate buffer stock solution.

## Tissue sample preparation: mouse brain, block of a fresh frozen human brain

6h

1 If the sample is a fresh frozen sample and requires sub-dissection, bring the tissue at -40 °C for more than 03:00:00. This enables the sub-dissection of the sample using a regular disposable blade on dry ice.

2 If the sample is frozen, bring the tissue at -20 °C for more than 03:00:00. This reduces the risk of the formation of ice crystals when moved to a fixation solution.

3 Gently shake tissue in 40 mL 4% paraformaldehyde in PBS Overnight at 4 °C. The amount of PFA should be adjusted based on the size of the tissue. It is usually advised to use at least 10 times the volume of the tissue sample.

> Note
>
> Forty milliliters is enough for six mouse brains.
> For more than six, I suggest preparing a separate reaction tube. We use a regular 50 mL tube for this reaction.

4 (Optional) Subdissect the tissue. The dissected volume will be the final volume.

5 Initiate dehydration by immersing the tissue in a gradient concentration of methanol (MeOH). 6h
The volume of the solution is the same as the volume used for PFA fixation.
Replace the solution with 80% MeOH. Gently shake the sample at 4 °C for 02:00:00.

Refresh 80% MeOH. Gently shake the sample at 4 °C for 02:00:00.

Replace the solution with 100% MeOH. Gently shake the sample at 4 °C for 02:00:00.

Refresh 100% MeOH. Gently shake the sample at Room temperature Overnight.

Note

80% MeOH at this step can be at either 4°C or RT. Empirically, we have not seen any difference in the performance.

6 Refresh 100% MeOH. Gently shake the sample at 50 °C for 120:00:00 (5 days).
Refresh MeOH every day but can be skipped during a weekend or national holidays. If necessary, the delipidated tissues can be stored at -20°C until use.

## (Optional) Photobleaching of autofluorescence 10h

7 4h
Though optional in rodent tissue, photobleaching will significantly improve the signal-to-noise ratio. This step is a must for human tissue.

Depending on the level of autofluorescence, prepare
#1, BABB, or
#2, [M] 0.2 % volume $H_2O_2$ in BAcBB.
BABB can be prepared by mixing benzyl alcohol and benzyl benzoate in a 1:2 ratio.
BAcBB can be prepared by mixing benzyl acetate and benzyl benzoate in a 1:1 ratio.
To mix $H_2O_2$ in BAcBB, add the required amount of $H_2O_2$ to Benzyl acetate, and very gently shake the solvent until fully mixed (Do NOT vortex!). Then add benzyl benzoate and mix.
#2 is preferred for tissue with strong autofluorescence, such as human tissue.
Otherwise, use #1.

Use 3 mL for a whole mouse brain or 2 mL if the tissue is smaller than that.
After 01:00:00 of shaking in the cleaning solution, refresh the solution and keep the tissue shaken until fully cleared. This usually takes 03:00:00 for a whole brain.

Once the tissue gets cleared, start photobleaching under strong LED illumination.
A large photobleaching device can be fabricated by referring https://github.com/NBelenko/Photobleacher.
If not, use an LED from Thorlabs (MWWHLP1) with an adjustable collimation adapter (SM1U25-A) and give max-power illumination from a 2-cm distance.

Illuminate the tissue Overnight .

8 Wash out BABB by immersing the tissue in 100% MeOH. 6h
Gently shake the tissue at Room temperature 02:00:00 .
Refresh 100% MeOH. Gently shake the tissue at Room temperature 02:00:00 .
Refresh 100% MeOH. Gently shake the tissue at Room temperature 02:00:00 .

If necessary, the delipidated tissues can be stored at -20°C until use.

## Pre-processing 4h 30m

9 We use a 5 mL tube with a screw cap for a whole-mouse brain or a 2 mL Eppentube for smaller tissue. 1h

1.5 mL of solution is used for a whole-mouse brain or 1 mL of solution for smaller tissue.

10 **(Optional)** 3h 30m
**We found that this protease step introduces significant variability depending on the size of tissue, the manufacturer, or batch of proteinase K. Therefore, we no longer recommend performing this proteinase K step, and it is entirely optional. You may proceed directly to the primary hybridization step following the MeOH wash described above.**

Transfer tissue in MeOH to MeOH with 2.5 M LiCl.
Gently shake the tissue for 01:00:00 at Room temperature .

Mix 10 ug/mL of proteinase K in primary hybridization buffer (1 M NaCl, 40% formamide in 100 mM lithium maleate buffer (pH 7.0)).

Place the tissue in the proteinase K solution, and gently shake the tissue for 03:00:00 at Room temperature .

Wash tissue with primary hybridization buffer at Room temperature . Wash for 00:30:00 and repeat the process in total twice.

Note

We no longer recommend performing this proteinase K step.

## Primary probe hybridization

2d 2h

11 1.5 mL of solution is used for a whole-mouse brain or 1 mL of solution for smaller tissue.
Replace the solution with hybridization buffer with primary probes.
Shake the sample gently at 37 °C for 48:00:00 or 2 nights. This can be 3 nights, depending on the weekend schedule.

2d

For the concentration of the primary probe, I advise beginning with [M] 4.0 nanomolar (nM) per each oligonucleotide.
If you see the limited penetration of the HCR probes, you should reduce the concentration of the primary probe. The primary probe penetrates evenly regardless of the concentration. Too high concentration of primary probes limits the penetration of HCR probes by trapping HCR probes at the rim.
Check out my GitHub repository (https://github.com/tatz-murakami/split-oligo-designer) for the design of the primary probes.

12 Wash the tissue with pre-HCR washing buffer (1 M NaCl and 20% formamide in 100 mM lithium maleate buffer (pH 7.0)) by gently shaking for 02:00:00 three times before the HCR amplification.

2h

## HCR probe hybridization

2d 2h

13 For HCR amplification, use 900 µL for a whole-mouse brain, or 600 µL for smaller tissue.
For washing, use 1.5 mL or 1.0 mL .

2d

Place the tissue in HCR buffer (1 M NaCl, 20% formamide, and 10% PEG(20000) in 100 mM lithium maleate buffer (pH ~7.0)) with 30 nM of HCR probes.
The HCR probes are denatured prior to the reaction by heating the probes to 95°C and leaving them at RT for at least 30 minutes.

Perform HCR reaction at 37 °C for 48:00:00 or 2 nights. This can be 3 nights, depending on the weekend schedule.
If you see the dim signals, extend the duration for 4 days with 60 nM of HCR probes.

Note

Please confirm that the average molecular mass of PEG is 20,000. We included the impact of the average molecular mass on the FISH signal in our original manuscript.

14 Wash the sample with post-HCR washing buffer (0.5 M LiCl, 40% formamide in 100 mM lithium maleate buffer (pH 7.0)) at Room temperature with a gentle shake for 02:00:00 three times. 2h

In our original manuscript, we used 1 M LiCl at 37 °C, but we found that 0.5 M LiCl at room temperature better preserves the signal without compromising the RNA quality. Please use the washing buffer described above.

## Tissue clearing 1h

15 1.5 mL of solution is used for a whole-mouse brain or 1 mL of solution for smaller tissue. 1h

Place the tissue in pre-clearing washing buffer (2.5 M LiCl in 100 mM lithium maleate buffer (pH 7.0)).
The tissue is gently shaken at Room temperature Overnight .

Replace the solution with 2.5 M LiCl MeOH solution.
Gently shake the sample for 00:30:00 at Room temperature .
Refresh 100% MeOH.
Gently shake the sample for 00:30:00 at Room temperature .
Refresh 100% MeOH.
Gently shake the sample for 00:30:00 at Room temperature .

Bring the tissue to BABB.
Gently shake the sample for 01:00:00 at Room temperature .
Repeat the process until the tissue becomes transparent. Usually, a whole brain can be cleared in three hours.

## Imaging

16 Image the sample as you like! I highly recommend the use of light-sheet microscopy designed for cleared tissue. I suggest the use of BABB as an immersion medium, but it is also possible to perform imaging using an immersion oil mixture, a 3:1 mixture of HIVAC-F4 (Shin-Etsu, refractive index = 1.555) and HIVAC-F5 (Shin-Etsu, refractive index = 1.575), if there is a need to avoid using BABB as the immersion medium.

## Photobleaching (Only for multi-round staining)

17 Please go to go to step #7 . Photobleach with $H_2O_2$ to bleach the fluorescent dyes.